

REMARKS

Claims 1, 2, 4-7, 10, 14, 15, 18, and 27-32 are pending in the application, claims 3 and 19-26 having been newly canceled and claims 27-32 added. Claims 1, 2, 6 and 7 are amended. The Examiner withdrew claims 10 and 18 from consideration. Claims 1, 2, 4-7, 14, 15, and 27-32 are under examination.

Support for the amendments to claims 1, 2, 6 and 7 can be found, for example, in claim 3 (now canceled) and at page 3, lines 30-32; page 9, lines 18-20; page 10, lines 21-27; page 11, line 21 to page 12, line 14; and page 12, lines 30-36. Support for new claims 27-32 can be found, e.g., at page 3, lines 27, to page 4, line 6; page 6, lines 7-15; page 8, lines 5-6 and 32-35; and page 14, lines 30-32; and in the claims as originally filed. The indicated passages from the specification disclose that the screening methods can ultimately be used to generate a bispecific antibody (BsAb) or a multi-specific antibody with specificity for three or more antigens. As such a use would be recognized by one of ordinary skill in the art as inherently involving the steps specified in the various new claims, Applicants submit that no new matter has been added. Applicants further submit that, since the new claims are all method claims that are dependent on method claims 1, 2, 5 or 7, the new claims belong to the same restriction group as the latter claims and should be examined in the present case.

Claims 1-7 and 13-15 were rejected for lack of enablement under 35 USC § 112, paragraph 1. See pages 4-7 of the Office action. The Office action acknowledged that one prior ground for rejection, that the host cell used with the phage-based screening assay must be bacterial, was overcome by Applicants' prior amendment of the claims to require use of bacterial host cells. The second ground for the enablement rejection appears to be based on the Examiner's concern that the claimed screening assay would not work even in bacterial hosts. The Office action says at page 5,

The specification is enabling for using a host cell system compatible with a phage library being introduced into the host cell such as a bacterium which produces phage particles expressing an assembled antibody heavy and light chain on the particle surface after following the method steps.... The specification is not enabling for any host cell having the ability to "secrete" an antibody heavy chain much less where a bacterial host

cell secretes the first or second or third antibody heavy chain. The claims encompass any host cell a) having the ability to secrete an antibody heavy chain and b) capable of being infected with a phage particle library whereas the specification is only enabling for using phage particles to infect a bacterium where at most the resultant, selected phage particle library also expresses the heavy chain and light on the surface.

As Applicants understand it, the Examiner's position seems to be that a phage-based screening assay in bacteria that produces phage particles expressing an assembled antibody heavy and light chain on the phage particle surface would work, while a phage-based screening assay that involves secretion of a heavy chain by the bacterium would not work. If this interpretation of the Examiner's position is not what was intended, Applicants respectfully request clarification.

Applicants point out that phage-based antibody screening assays that involves association of separate heavy and light chains (or fragments thereof) on the surface of the phage generally require that both of the chains be secreted in the periplasmic space (one of the chains being fused to a phage protein that self-assembles with other phage proteins to form the phage particle and the second chain being expressed as a separate polypeptide that associates with the phage-anchored chain). This is illustrated by the Griffiths et al. reference (EMBO J 13:3245-3260, 1994) that was supposed to be submitted with the prior Response filed March 17, 2009. A copy of the Griffiths et al. reference is attached hereto as Exhibit A; Applicants sincerely apologize for neglecting to include it with the prior Response. Griffiths et al. describe a method of screening for two-chain antibody fragments (Fab fragments) using phage display in bacteria. See the abstract. In the Griffiths et al. scheme illustrated in Fig.2A, both the heavy chain fragment (VH+CH1) and the light chain fragment (C κ) are encoded as separate polypeptides on a phage vector, with the light chain fragment linked to the phage gene III leader sequence and the heavy chain fragment linked to both the PelB leader sequence and the phage gene III polypeptide (see Fig. 2A). The heavy chain fragment would thus be secreted into the periplasmic space by the PelB leader sequence as a fusion protein linked to the phage gene III polypeptide. The light chain fragment is presumably secreted into the periplasmic space by the gene III leader sequence. Though Griffiths et al. does not specify where the association of the two fragments to form a Fab occurs, presumably it is in the periplasmic space where the phage structure

(containing the gene III protein) is assembled. Thus, Griffiths et al. provides evidence that a functional Fab fragment can form in the periplasmic space following expression and secretion of two antibody chain fragments, one being attached to a phage protein.

Furthermore, Kipriyanov (cited in the Office action) states at page 181, left column, that “bacterial systems are widely used for the production of antibody fragments such as Fab...” and “A rapid growth in the field of antibody engineering occurred after it was shown that functional antibody fragments could be secreted into the periplasmic space and even into the medium of *E. coli* by fusing a bacterial signal peptide to the antibody’s N-terminus.” Furthermore, Kipriyanov says at page 190, “Periplasmic expression has permitted the functional testing of a wide variety of antibody fragments with different antigen binding specificities. The antibody fragments are usually correctly processed in the periplasm, they contain intramolecular disulfide bonds and are soluble.” Since the Examiner indicated at page 6 of the Office action that reliance on the teachings of Kipriyanov in this regard would require inclusion of a claim limitation regarding the use of a bacterial secretion signal sequence, Applicants have added such a limitation to the claims. Though Kipriyanov goes on to say that “high-level expression of a recombinant protein with a bacterial signal sequence in *E. coli* often results in the accumulation of insoluble antibody fragments after transport to the periplasm,” this particular problem is said to occur with “high-level” expression, as when large amounts of a given antibody are desired, and not the level of expression one would seek for “functional testing” (e.g., in a screening assay), as disclosed by Kipriyanov. Applicants believe that Kipriyanov is strong support for Applicants’ position that the present claims are fully enabled.

The Office action also cites Zauderer et al., US 20080167193, in support of the rejection, referring in particular to passages at [0013] and [0016] of this reference. Zauderer et al., which touts antibody libraries expressed in eukaryotic cells, says of bacteriophage and bacterial libraries:

Many antigen binding regions, however, are difficult to assemble correctly as a fusion protein in bacterial cells. In addition, the protein will not undergo normal eukaryotic post-translational modifications. As a result, this method imposes a different selective filter on the antibody specificities that can be obtained... . Assays for expression in bacterial hosts are often impeded, however, because the protein may not be sufficiently

expressed in bacterial hosts, it may be expressed in the wrong conformation, and it may not be processed, and/or transported as it would in a eukaryotic system. Many of these problems have been encountered in attempts to produce immunoglobulin molecules in bacterial hosts. Zauderer et al., [0013] and [0016].

Regardless of whether the concerns stated by Zauderer et al. above are true, it remains a fact that bacteriophage display of antibody fragments has long been a standard technique in the field. See, for example, the many publications cited in Zauderer et al. at paragraph [0011]. Clearly the methods generally work and can be carried out successfully without undue experimentation. There is absolutely no reason to conclude that Zauderer et al.'s unsupported statements about "difficulties" and "problems" suddenly remove an otherwise standard technique from the skill set of those of ordinary skill in the art. Nor is there any reason to believe that Applicants' novel screening method would be any more difficult to carry out than the phage display techniques that are standard in the art.

Applicants submit that the presently claimed methods are fully enabled by the specification. Withdrawal of the rejection and allowance of the claims is respectfully requested.

Please apply the fee necessary for the three-month extension of time, as well as any other charges or credits, to deposit account 06-1050, referencing Attorney Docket No. 14875-0148US1.

Respectfully submitted,

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Isolation of high affinity human antibodies directly from large synthetic repertoires

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Antibody fragments of moderate affinity ($\sim\mu\text{M}$) can be isolated from repertoires of 10^8 immunoglobulin genes by phage display and rounds of selection with antigen, and the affinities improved by further rounds of mutation and selection. Here, as an alternative strategy, we attempted to isolate high affinity human antibodies directly from large repertoires. We first created highly diverse repertoires of heavy and light chains entirely *in vitro* from a bank of human V gene segments and then, by recombination of the repertoires in bacteria, generated a large (close to 6.5×10^{10}) synthetic repertoire of Fab fragments displayed on filamentous phage. From this repertoire we isolated Fab fragments which bound to a range of different antigens and haptens, and with affinities comparable with those of antibodies from a secondary immune response in mice (up to 4 nM). Although the VH-26 (DP-47) segment was the most commonly used segment in both artificial and natural repertoires, there were also major differences in the pattern of segment usage. Such comparisons may help dissect the contributions of biological mechanisms and structural features governing V gene usage *in vivo*.

Key words: high affinity/human antibodies/phage

Introduction

The display on the surface of filamentous bacteriophage (Smith, 1985) of antibody fragments (McCafferty *et al.*, 1990; Barbas *et al.*, 1991; Breitling *et al.*, 1991; Garrard *et al.*, 1991; Hoogenboom *et al.*, 1991) by fusion to a

minor coat protein of phage (pIII), and selection of the phage with antigen, has provided a powerful means of making antibodies of predefined binding specificity from V gene repertoires (for review see Winter *et al.*, 1994). Starting from repertoires derived from the V genes of an immune response, antibody fragments have been readily isolated with high affinity (Clackson *et al.*, 1991), and even with neutralizing activities against virus (Barbas *et al.*, 1992a,b).

The display and selection of antibody fragments on the surface of phage mimics immune selection (for review see Marks *et al.*, 1992a), and antibodies have also been isolated without immunization, from repertoires of V genes rearranged *in vivo* (Marks *et al.*, 1991) or *in vitro* (Hoogenboom and Winter, 1992). The same phage repertoire may be used to generate many different binding specificities, including those that are difficult to raise by immunization, for example against self-antigens (Griffiths *et al.*, 1993; Marks *et al.*, 1993) or proteins of the lumen of the endoplasmic reticulum (Nissim *et al.*, 1994). Antibodies from such 'single pot' libraries appear to be highly specific and have been used for 'on-line detection' of antigen in biosensors (M.Malmqvist, unpublished), and for Western blotting and epitope mapping (Nissim *et al.*, 1994). But the affinities were not high ($\sim\mu\text{M}$), as expected for primary repertoires of 10^7 – 10^8 clones.

It should also be possible to isolate high affinity antibodies (<10 nM) directly from primary repertoires of sufficiently large diversity and size (Perelson and Oster, 1979), but the size and diversity of repertoires required is not known. The size and 'shape' of natural repertoires is limited not only by the number of B-lymphocytes ($\sim 5 \times 10^8$ in mouse and $\sim 10^{12}$ in human), and the number of lymphocytes in each clone, but also by the processes of clonal deletion and anergy that lead to self tolerance (for review see Nossal, 1993).

Here we constructed a highly diverse combinatorial repertoire entirely *in vitro*, using V gene segments as building blocks. We used the vast majority of heavy and light chain segments used *in vivo* and encoded part, or all, of each CDR3 loop by random sequence. To make the repertoire as large as possible we infected bacteria harbouring a 'donor' heavy chain repertoire (on a plasmid) with an 'acceptor' light chain repertoire (on phage). The two chains were combined on the same (phage) replicon within the bacterium by Cre catalysed recombination at *loxP* sites. This process, termed 'combinatorial infection' (Waterhouse *et al.*, 1993), generates a large number of heavy and light chain combinations, potentially as large as the number of bacteria that have been infected. The repertoire was characterized by the properties of the selected Fab fragments.

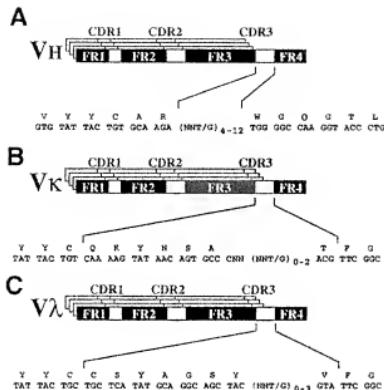


Fig. 1. Construction of synthetic heavy and light chain repertoires. (A) The repertoire of heavy chains ($>10^9$ different clones) was built from 49 cloned V_H segments (Tomlinson *et al.*, 1992; Nissim *et al.*, 1994), with CDR3 loops of four to 12 residues of random sequence. (B) The repertoire of V_L light chains (9×10^9 clones) was built from 26 cloned V_L segments (Cox *et al.*, 1994) with CDR3 loops of eight to 10 residues that included one, two or three residues of random sequence in all cases. DPK-12 is shown by way of example. (C) The repertoire of V_λ light chains (7.4×10^9 clones) was built from 21 cloned V_λ segments (Williams and Winter, 1993), with CDR3 loops of eight to 13 residues that included zero, one, two, three, four or five residues of random sequence. DPL-12 is shown by way of example. CDR, complementarity determining region; FR, framework region. Neither the single segment of the V_L 7 subgroup, nor the few segments from the V_L families 4, 5 or 6, were included in the repertoire. Segments rarely used *in vivo*, for example the chain segments (DPK-2, -7, -10, -12, -17, -19, -20, -23 and -28) from the diverse V_L locus (Cox *et al.*, 1994), were represented equally in the synthetic repertoire. Some of the V_H gene segments (DP-1, -12, -30, -39, -40, -44, -45 and -69) included in the repertoire are now known to be located on chromosomes 15 or 16, and therefore outside the functional locus (Tomlinson *et al.*, 1994).

Results

Repertoire size and diversity

Heavy and light chain repertoires were built from the majority of human V gene segments as described in Figure 1. The light chain repertoire was cloned into 'acceptor' fd phage (tetracycline resistant), with a 'dummy' heavy chain (Figure 2A), and the heavy chain repertoire into 'donor' plasmid (ampicillin resistant) (Figure 2B). The repertoires, $>10^9$ heavy chains and $>8 \times 10^9$ light chains, were combined by infection of *Escherichia coli* harbouring the donor heavy chains with fd phage carrying the light chains. If every heavy chain was paired with every light chain, this would generate a repertoire of $>8 \times 10^{13}$ antibodies. The culture was then co-infected with bacteriophage P1 (chloramphenicol resistant), which provides the Cre recombinase (Waterhouse *et al.*, 1993), leading to 6.5×10^{10} colonies resistant to ampicillin, tetracycline and chloramphenicol (for summary, see Table 1). After growth of the cultures, the fd phage were used to infect *E. coli*. Twenty-eight per cent of the acceptor phage were shown to have acquired a heavy chain from the donor vector (see

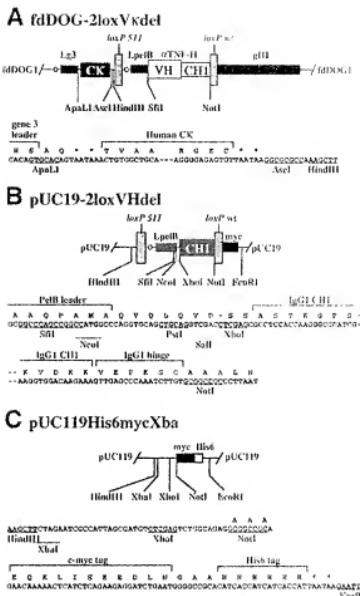


Fig. 2. Vectors. (A) The fd phage 'acceptor' vector fdDOG-2loxVkdelf. Light chain genes (V_H-C_X and V_L-C_λ) are cloned into this vector as ApaLI-Axel fragments. (B) The plasmid 'donor' vector pUC19-2loxVHdel. Heavy chain variable region genes (V_H genes) are cloned into this vector as NcoI-XbaI fragments. (C) The plasmid expression vector pUC119His6mycXba. Heavy and light chain genes encoding Fabs are cloned into this vector as XbaI-NcoI fragments. Other features are marked as follows: sequences encoding Lg3, gene III leader sequence; LpeB, pIII leader sequence; Cx, human kappa light chain constant region; VH, heavy chain variable region; CH1, first heavy chain constant domain (human Cx1 in fdDOG-2loxVkdelf and human Cx1 in pUC19-2loxVHdel); gIII, fd phage gene III; loxP wt, wild-type one point mutation (Hoess *et al.*, 1982); loxP 571, a mutant loxP site with a single point mutation (Hoess *et al.*, 1986); myc, peptide from c-myc recognized by the monoclonal antibody 9E10 (Munro and Pelham, 1986); His6, six histidines.

Materials and methods). As there are multiple copies of plasmid and phage replicons in each bacterial cell when Cre recombinase is delivered by phage P1 infection and at least 60 phage are produced per bacterium after overnight growth, we believe that each bacterium should yield at least one phage containing the heavy chain from the donor vector and therefore we estimate that the repertoire contained close to 6.5×10^{10} different phage antibodies, with up to 60 copies of each.

Specificity of selected antibodies

The repertoire of phage was selected with a range of antigens, leading to isolation of binding specificities, as

Table I. Determination of repertoire size

Sample Point	Total no. of colony forming units ($\times 10^{10}$)						Significance
	No antibiotic	amp ^R	ter ^R	chlor ^R	amp ^R + ter ^R	amp ^R + chlor ^R	
1	0.17						size of pUC19-2IoxVHlib inoculum from frozen stock:
2	3.4	2.3					2.3 $\times 10^9$
3	65 (λ)	10 (λ)	19 (λ)				no. of <i>E.coli</i> containing pUC19-2IoxVHlib from overnight culture:
	73 (λ)	11 (λ)	8.0 (λ)				1.9 $\times 10^1$ (λ)
4	9.5 (λ)	22 (λ)	4.5 (λ)	6.0 (λ)	3.0 (λ)	2.0 (λ)	no. of <i>E.coli</i> containing pUC19-2IoxVHlib infected with fdDOG-2IoxVHlib:
	29 (λ)	28 (λ)	7.5 (λ)	28 (λ)	3.0 (λ)	4.5 (λ)	8.0 $\times 10^0$ (λ)
5							no. of <i>E.coli</i> containing pUC and fdDOG replicons co-infected with phage P1:
							3.0 $\times 10^0$ (λ)
							titre of fdDOG phage (t.u.) immediately after P1 infection and centrifugation:
6	31 (λ)			16 (λ)			9.9 $\times 10^0$ (λ)
7	35 (λ)			9.5 (λ)			1.2 $\times 10^1$ (λ)
							1.8 $\times 10^1$ (λ)
							9.5 $\times 10^0$ (λ)
							1.1 $\times 10^1$ (λ)
							3.0 $\times 10^0$ (λ)

Sample point, see Materials and methods; amp^R, ampicillin resistant; ter^R, tetracycline resistant; chlor^R, chloramphenicol resistant.

summarized in Table II. The repertoire was selected on all antigens and haptens by panning on antigen coated immunoblates; for the haptens NIP and fluorescein, the phage were also captured with biotinylated NIP-BSA and FITC-BSA and streptavidin-coated paramagnetic beads. To follow the selection process, *E.coli* were infected with the eluted phage after each round, and the phage screened for binding to antigen by ELISA, either as a 'polyclonal' population or as phage clones. DNA encoding the Fab fragments was amplified using the polymerase chain reaction from the population of phage after two to four rounds of selection and recloned into plasmid (Figure 2C) for expression of soluble Fab fragments.

We focused on the characterization of Fab fragments with specificities against the haptens-BSA conjugates of NIP (3-iodo-4-hydroxy-5-nitrophenyl-acetate) and FITC (fluorescein 5-isothiocyanate), as it would allow comparisons with the natural immune response to both haptens in mice, and facilitate the measurement of binding affinities to the free haptens (by fluorescence quench titrations). The fragments against haptens-BSA were first screened for binding to the free haptens by competition with fluorescein or NIP- α -caproic acid (NIP- α CA), or for binding to BSA. Most of the Fab fragments bound to haptens-BSA, but some also bound to BSA, or to free haptens.

We also characterized Fab fragments against four antigens of the kringle-serine protease family—hepatocyte growth factor/scatter factor (HGF/SF), plasmin, urokinase-type plasminogen activator (u-PA), and tissue-type plasminogen activator (t-PA)—as members of a family of related proteins. Binding of 'polyclonal' phage was detected after three rounds of selection and proved to be specific, despite homologies between the members of this family (Figure 3). We also characterized Fab fragments against the mouse monoclonal antibody NQ11/7.22 (Griffiths *et al.*, 1984), as the binding could be mapped to the variable regions, to the Fc region and to the remaining portions of the monoclonal antibody (Table III).

Segment usage of selected heavy and light chains

The phage selected from the large (6.5×10^{10} clones) repertoire by binding to antigen were characterized by DNA sequencing. Many of the clones were different: 137

Table II. Binding specificities isolated from the library

Haptens
3-iodo-4-hydroxy-5-nitrophenyl-acetate (NIP)
Fluorescein
2-phenylloxazol-5-one (phOx)
N-[Carboxymethyl]-4-[(<i>p</i> -nitrobenzyloxyphosphoryl)-butyramide]
N-[2-hydroxy-3-(4-nitrophenyl)]-propyl-L-prolyl-glycine
Foreign antigens
Serum albumin (BSA) (bovine)
Tubulin (bovine)
Caldmodulin (bovine)
Hepatocyte growth factor/scatter factor (HGF/SF) (murine)
Monoclonal antibody NQ11/7.22 (murine)
FixL (<i>Bradyrhizobium japonicum</i>)
Acetolactate synthase (ALS) (<i>Brassica napus</i>)
Lol p1 (<i>Lolium perenne</i>)
Gene product of CDC4 (<i>Schizosaccharomyces pombe</i>)
Gene product of CDC8 (<i>Schizosaccharomyces pombe</i>)
Maltose binding protein (<i>E.coli</i>)
gp120 (HIV-1)
gp11 (T4 phage)
gp9 (T4 phage)
Human antigens
Tumour necrosis factor α (TNF α)
Thyroglobulin
High affinity IgE receptor (Fc ϵ RI)
Tissue-type plasminogen activator (t-PA)
Urokinase-type plasminogen activator (u-PA)
Plasmin
Carcinoembryonic antigen (CEA)
c-erb B2
Tau40
Elongation factor 1 α (EF-1 α)
Calreticulin
Calnexin
Ferritin light chain
Factor VIII
U1 srRNA
U1A protein
U1C protein

unique antigen-binding Fab fragments (with differing light or heavy chain protein sequences) were identified from a total of 215 clones sequenced (Table III). A range of V gene segments was seen: 17 of the 49 V_H segments, 10 of the 26 V_k segments and nine of the 21 V_{λ} segments

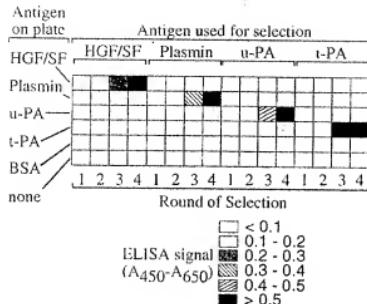


Fig. 3. Specificity of polyclonal phage against kringle serine protease family. 'Polyclonal' phage after one, two, three and four rounds of selection on either HGF/SF, plasmin, u-PA or t-PA, were assayed by ELISA for binding to the other members of the family, and to BSA.

(see Figure 4). Each of the major heavy and light chain families were represented (Chuchana *et al.*, 1990; Kabat *et al.*, 1991; Williams and Winter, 1993), but not all the minor families. Thus V_H segments were seen from families 1, 3, 4 and 5, but not 2 and 6; V_{κ} segments from subgroups 1, 2, 3 and 4, but not from 6; and V_{λ} segments from families 1, 2, 3, 7 and 8, but not 9. The heavy chain V_H segment DP-45 (included in the repertoire, but located on chromosome 16 outside the major locus on chromosome 14) was found in two Fab fragments (Table III and Figure 4A) binding to NIP-BSA.

Some V gene segments (V_H segments DP-7, DP-38, DP-47 and DP-67; V_{κ} segment DPK-15; and V_{λ} segment DPL-3) were seen frequently in the synthetic chains: of these only the V_H segment DP-47 is common in natural antibodies. Conversely, some segments (like V_H segments DP-63 and DP-71; V_{κ} segments DPK-1 and DPK-21; and V_{λ} segments DPL-5 and DPL-23) that are common in natural antibodies were not seen in the synthetic chains (Figure 4). Thus, except for DP-47, the pattern of usage of the segments from the synthetic repertoire, summed

Table III. CDR3 sequences and germline V gene segments from antigen-binding clones

Antigen ^a	Clone	Heavy chain ^b			Light chain ^c			Selection method ^d	Round ^e	No. of copies ^f
		Family	Segment	CDR3 ^g	Family	Segment	CDR3 ^g			
NIP-BSA	G09	VH3	DP-38	AGTL	V λ 1	DPL-3	AAWDDDSLVL	M	4	1
NIP-BSA	E01	VH3	DP-38	AGTL	V λ 2	DPL-12	MQSILQLPT	M	3/4	2
NIP-BSA	G10	VH3	DP-38	AGTL	V λ 2	DPL-12	MQSILQLPAT	M	4	1
NIP-BSA	G04	VH3	DP-38	AGTL	V λ 1	DPL-3	AAWDDGDSLVL	M	4	1
NIP-BSA	H08	VH3	DP-38	AGTL	V λ 1	DPL-3	AAWDDGSLGV	M	4	1
NIP-BSA	G07	VH3	DP-38	AGTL	V λ 3	DPL-16	NSRDSGSVVRV	M	4	1
NIP-BSA	C09	VH3	DP-38	GGKD	V λ 7	DPL-16	LLYYGQAYV	Im	4	1
NIP-BSA	F03	VH1	DP-10	GGRRL	V λ 3	DPL-16	NSRDSGSVSRV	M	3	1
NIP-BSA	E07	VH3	DP-38	GOTQ	V λ 1	DPL-3	AAWDDSLPVV	M	4	1
NIP-BSA	H05	VH3	DP-38	GOTQ	V λ 1	DPL-3	AAWDDSLCPFV	M	4	1
NIP-BSA	H03	VH3	DP-38	HQHQH	V λ 1	DPL-3	AAWDDSLAWFV	M	4	1
NIP-BSA	H02	VH3	DP-38	KGSE	V λ 1	DPL-4	AAWDDSLVW	M	4	1
NIP-BSA	A12	VH3	DP-47	KGWS	V λ 1	DPL-4	LAWDDTSRWW	Im	3	1
NIP-BSA	A10	VH1	DP-47	KLWS	V λ 1	DPL-2	TAWDDSLAVV	Im	3	1
NIP-BSA	D08	VH3	DP-47	KLWS	V λ 3	DPL-16	NSRDSGSNHRV	Im	4	1
NIP-BSA	G02	VH3	DP-49	LGKA	V λ 3	DPL-22	QYQYGT	M	4	1
NIP-BSA	E06	VH3	DP-38	NGYF	V λ 1	DPL-3	AAWDDSLRLV	M	3	1
NIP-BSA	D03	VH3	DP-49	PROY	V λ 1	DPL-3	AAWDDSLRLVY	Im	4	1
NIP-BSA	B02	VH3	DP-46	MYMRS	V λ 2	DPL-18	MOGTHWRPPT	Im	3	1
NIP-BSA	E02	VH3	DP-46	MYRSV	V λ 2	DPL-18	MOGKHWPLT	M	3	1
NIP-BSA	A06	VH3	DP-42	NGGHIV	V λ 1	DPL-3	AAWDDSLGLF	Im	3	1
NIP-BSA	D05	VH3	DP-47	PAGSSE	V λ 2	DPL-18	MQGTHRRAT	Im	4	1
NIP-BSA	A04	VH3	DP-38	PATRS	V λ 2	DPL-15	MQALQTPLT	Im	3	1
NIP-BSA	F04	VH3	DP-47	PFATP	V λ 2	DPL-18	MRGTHRRAT	M	3	1
NIP-BSA	E08	VH3	DP-51	PLAII	V λ 2	DPL-18	MQGTHWHPT	M	3	1
NIP-BSA	C05	VH3	DP-32	PLGHA	V λ 2	DPL-15	MQGTHWHPT	Im	4	1
NIP-BSA	E12	VH3	DP-47	PMRGV	V λ 2	DPL-18	MQGTHRRAT	M	3	1
NIP-BSA	E03	VH3	DP-38	PNGDQ	V λ 1	DPL-3	AAWDDSLA-FV	M	3	1
NIP-BSA	E09	VH3	DP-38	POTRRE	V λ 2	DPL-15	MQALQTPT	M	3	1
NIP-BSA	A08	VH3	DP-47	PRLPR	V λ 1	DPL-9	QQSYSTR	Im	3	1
NIP-BSA	E10	VH3	DP-73	PGSNNV	V λ 2	DPL-19	MQGTHWPPT	M	3	1
NIP-BSA	A05	VH1	DP-25	QGRLRN	V λ 2	DPL-15	MQALQTPLT	Im	3	1
NIP-BSA	D06	VH3	DP-47	RGHKA	V λ 2	DPL-18	MQGTHWPAT	Im	4	1
NIP-BSA	D02	VH3	DP-51	SDDPS	V λ 1	DPL-3	AAWDDSLRSV	Im	4	1
NIP-BSA	F01	VH3	DP-47	TFSPQ	V λ 2	DPL-18	MQGTHWPAT	M	3	1
NIP-BSA	B03	VH3	DP-47	SFRRNL	V λ 1	DPL-3	AAWDDSLA-FV	Im	3	1
NIP-BSA	A11	VH3	DP-58	SFRRNL	V λ 3	DPL-16	NSWDNSLSPV	Im	3	1
NIP-BSA	C03	VH3	DP-38	PGYRGR	V λ 2	DPL-15	MQALQSPT	Im	4	2
NIP-BSA	D07	VH3	DP-38	PGYRGR	V λ 2	DPL-12	MQSLQPLT	Im	4	1
NIP-BSA	D01	VH3	DP-38	PGYRGR	V λ 2	DPL-15	MQALQSPAT	Im	4	1
NIP-BSA	C10	VH3	DP-38	PGYRGR	V λ 2	DPL-15	MQALQTPVT	Im	4	1
NIP-BSA	C11	VH3	DP-38	PGYRGR	V λ 1	DPL-3	AAWDDSLAYV	Im	4	1
NIP-BSA	F04	VH3	DP-47	PKNQCR	V λ 3	DPL-16	NSRDSGSRVNV	M	3	1
NIP-BSA	B04	VH3	DP-47	PKRQTY	V λ 2	DPL-15	MQALRTRT	Im	3	1
NIP-BSA	F05	VH3	DP-38	VNSRFAT	V λ 3	DPL-16	NSRDSGSVSRV	M	3	1
NIP-BSA	E11	VH4	DP-67	IKFRSSSI	V λ 2	DPL-19	MQGTHWPPT	M	3	1
NIP-BSA	H06	VH4	DP-67	SFAKAFDY	V λ 1	DPL-3	AAWDDSLPYV	M	4	1

Table III. Continued

Antigen ^a	Clone	Heavy chains ^b			Light chains ^c			Selection method ^d	Round ^e	No. of copies ^f
		Family	Segment	CDR3 ^g	Family	Segment	CDR3 ^g			
NIP-BSA	E09	VH4	DP-67	SFAKAFDY	V _λ 3	DPL-16	NSRDSSGSRV	M	3	1
NIP-BSA	C02	VH1	DP-7	SKTRIFSDY	V _κ 2	DPK-18	MQGTHWHPPT	Im	4	1
NIP-BSA	G03	VH3	DP-47	SFLSKFEDY	V _λ 3	DPL-16	NSRDSSGSRV	M	4	1
NIP-BSA	A07	VH1	DP-47	SYVYVADY	V _λ 3	DPL-3	AAWDSSLFPV	Im	3	1
NIP-BSA	C03	VH3	DP-45	SYMMGRMRN	V _λ 3	DPL-3	MQGTHWHRV	Im	4	1
NIP-BSA	A02	VH3	DP-42	HRRAYYMP	V _κ 2	DPK-18	MQGTHWHPPT	Im	3	1
NIP-BSA	A09	VH4	DP-67	IGKLSQLPTS	V _κ 2	DPK-18	MQGTHWHPPT	Im	5	1
NIP-BSA	E03	VH3	DP-47	RSGVRLMLD	V _κ 2	DPK-18	MQGTHWRT	M	3	1
NIP-BSA	A03	VH3	DP-42	HLRWASGGP	V _κ 2	DPK-18	MQGTHWRT	Im	3	1
NIP-BSA	G11	VH3	DP-47	PLNSKKNNTTQ	V _λ 1	DPL-3	AAWDSSLFV	M	4	1
NIP-BSA	C12	VH3	DP-49	GRTWSPSLPLR	V _κ 2	DPK-12	MQSHOLPLT	Im	4	1
NQ11 (Fc)	NMLL7	VH3	DP-47	KWGG	V _λ 1	DPL-2	AAWDSSLGGSV	Im	2	1
NQ11 (Fc)	NMLL8	VH3	DP-14	FTGLDGL	V _κ 2	DPL-10	CYAGGSSYY	Im	2	1
NQ11 (Fc)	NMLL8	VH3	DP-47	KFGNPA	V _λ 3	DPL-23	QQDYNLLNT	Im	2	1
NQ11 (Fc)	NMLL3	VH3	DP-7/17	ASSEVYLO	V _λ 3	DPL-21	VALVYNGSASV	Im	2/3/4	25
NQ11 (Fc)	NMLL5	VH1	DP-10	SKLSTPVD	V _κ 2	DPL-13	MORIEFPT	Im	2	1
NQ11 (Fc)	NMLL2	VH3	DP-47	RSWDGGMVD	V _κ 1	DPL-5	AAWDSSLACAV	Im	2/3	4
NQ11 (Fc)	NMLL1	VH3	DP-3	SKLWVTFDY	V _λ 1	DPK-8/9	AAWDSSLRPT	Im	3	1
NQ11 (Fc)	NMLL6	VH3	DP-3	SKLWVTFDY	V _λ 1	DPL-2	AAWDSSLRPTV	Im	2/3/4	34
NQ11	NMLL4	VH3	DP-3	AKQSGVECLT	V _λ 1	DPL-3	AAWDSSLNV	Im	2	1
FTTC-BSA	B01	VH3	DP-47	SKYPLAWTLS	V _λ 1	DPL-2	AAWDSSLRNV	Im	2	1
FTTC-BSA	B06	VH3	DP-47	ALRR	V _κ 2	DPK-15	MQVLTQRT	Im	3	1
FTTC-BSA	A03	VH3	DP-47	IGQF	V _κ 2	DPK-15	MQALQRT	Im	3	1
FTTC-BSA	D10	VH3	DP-47	KAKT	V _κ 2	DPK-15	AAWDSSLAFV	Im	3/4	4
FTTC-BSA	G06	VH3	DP-47	KSAI	V _κ 2	DPK-15	MQALQRT	Im	4	1
FTTC-BSA	H03	VH3	DP-47	KSRW	V _κ 1	DPL-4	QOSYSTRM	M	4	1
FTTC-BSA	D12	VH3	DP-47	KSTV	V _κ 2	DPK-15	MQALRTT	Im	4	1
FTTC-BSA	A08	VH3	DP-47	LNRK	V _κ 2	DPK-15	MQALQRT	Im	3/4	4
FTTC-BSA	D09	VH3	DP-47	RHGS	V _κ 2	DPK-15	MQALQRT	Im	4	1
FTTC-BSA	G07	VH3	DP-47	RKRH	V _κ 2	DPK-15	MQALQTLT	M	4	1
FTTC-BSA	H02	VH3	DP-47	RSKT	V _κ 2	DPK-15	MQALQRT	M	4	1
FTTC-BSA	B02	VH3	DP-47	RWSF	V _λ 1	DPL-3	AAWDSSLV	M	4	1
FTTC-BSA	E11	VH3	DP-47	AKFRL	V _κ 2	DPK-15	MQALRTT	M	3	1
FTTC-BSA	C02	VH3	DP-47	AYHGR	V _κ 2	DPK-15	MQALQRT	M	3	1
FTTC-BSA	B02	VH3	DP-47	DPVLG	V _κ 2	DPK-15	MQALQRT	Im	4	1
FTTC-BSA	E07	VH3	DP-47	OKVLG	V _κ 2	DPK-15	MRAQLTPT	Im	3/4	2
FTTC-BSA	E09	VH3	DP-47	GSSRT	V _λ 1	DPL-3	AAWDSSLPGYV	M	3	1
FTTC-BSA	E04	VH3	DP-47	KRMDG	V _κ 2	DPK-15	MQALQRT	M	3	1
FTTC-BSA	A10	VH1	DP-10	LKRGH	V _λ 1	DPL-3	AAWDSSLGFV	Im	3	1
FTTC-BSA	D09	VH3	DP-47	LREY	V _κ 2	DPK-15	MQALRTT	Im	4	1
FTTC-BSA	G12	VH3	DP-47	RAGRD	V _λ 1	DPL-3	AAWDSSLFLV	M	4	1
FTTC-BSA	D01	VH3	DP-47	LKSAYK	V _κ 2	DPK-15	MQALQPTT	Im	4	1
FTTC-BSA	C12	VH3	DP-47	LNVRPK	V _κ 2	DPK-15	MQALQRT	Im	4	1
FTTC-BSA	B10	VH3	DP-47	SRGSK	V _κ 2	DPK-15	MQALQRT	Im	3	1
FTTC-BSA	E09	VH3	DP-47	SRGSKQSAAR	V _κ 2	DPK-15	MQALQRTT	M	3	1
FTTC-BSA	B03	VH4	DP-67	KTRRFRDY	V _κ 2	DPK-15	MQALQRT	M	3	1
FTTC-BSA	C01	VH4	DP-67	KTRRFRDY	V _κ 2	DPK-15	MQALQRT	M	3/4	2
FTTC-BSA	B09	VH3	DP-47	LKTSTPV	V _κ 2	DPK-15	MQALQRT	M	4	1
FTTC-BSA	G10	VH3	DP-47	LSRAFTM	V _κ 2	DPK-15	MQALQRTT	M	3	1
FTTC-BSA	E01	VH3	DP-47	LSRAFTM	V _κ 2	DPK-15	MQALQRT	M	3	1
FTTC-BSA	B07	VH4	DP-67	AQKRYQYDF	V _κ 2	DPK-12	MQSQLQRT	Im	3	1
FTTC-BSA	D01	VH4	DP-67	DLRKHFHDY	V _κ 1	DPL-9	QOSYSTRM	Im	4	1
FTTC-BSA	E10	VH4	DP-67	DRWYRVPFDY	V _λ 1	DPL-2	AAWDSSLIV	M	3	1
FTTC-BSA	A09	VH1	DP-14	GKGLRLFDY	V _λ 1	DPL-3	AAWDSSLV	Im	3	1
FTTC-BSA	Q08	VH3	DP-47	MQKQYQSAAR	V _κ 2	DPK-19	MQALQWHPPT	M	4	1
FTTC-BSA	B03	VH4	DP-67	KTRRFRDY	V _κ 2	DPK-15	MQALQHTRT	Im	3	1
FTTC-BSA	C01	VH4	DP-67	SYHRTFDY	V _κ 1	DPK-15	MQALQHT	Im	4	1
FTTC-BSA	G11	VH3	DP-47	PYAKDFTY	V _κ 2	DPK-15	MQALQHT	M	4	1
FTTC-BSA	G03	VH3	DP-47	RFARSFDY	V _κ 2	DPL-16	NSRDSSGSV	M	4	1
FTTC-BSA	A09	VH4	DP-67	RSFVGYE	V _λ 1	DPL-3	AAWDSSLV	Im	3	2
FTTC-BSA	D06	VH4	DP-67	TRWGRTFDY	V _κ 2	DPK-15	MQALQTR	Im	4	1
FTTC-BSA	C09	VH1	DP-7	SQKRLITG	V _κ 2	DPK-15	MQALQTR	Im	4	1
FTTC-BSA	C01	VH1	DP-7	SQKRLITG	V _κ 3	DPL-22	QOQYSPPT	Im	4	1
FTTC-BSA	H08	VH4	DP-67	SRKRAFDY	V _κ 2	DPK-15	MQALQTR	M	4	1
FTTC-BSA	C08	VH4	DP-67	SWVSGFDY	V _κ 1	DPL-9	QOQYSPPT	Im	4	2
FTTC-BSA	D04	VH1	DP-7	SYHRTFDY	V _κ 1	DPK-5	QOQNSFAAT	Im	4	1
FTTC-BSA	C05	VH1	DP-7	SYHRTFDY	V _κ 1	DPL-5	QOQNSFAAT	Im	4	1
FTTC-BSA	D05	VH1	DP-7	THSKTFDY	V _λ 3	DPL-21	VQVYVQVYVYV	Im	4	1
FTTC-BSA	B12	VH3	DP-47	TRKSSYGE	V _κ 2	DPK-15	MQALRTT	Im	3	1
FTTC-BSA	B04	VH4	DP-66	WSRRETNSY	V _λ 1	DPL-3	AAWDSSLWAV	Im	3	1
FTTC-BSA	A07	VH3	DP-47	RTGRALPRN	V _λ 1	DPL-3	AAWDSSLPLRRV	Im	3	1
FTTC-BSA	A02	VH3	DP-47	YRFYSPAPRD	V _λ 1	DPL-3	AAWDSSLPSGV	Im	3	1
FTTC-BSA	E04	VH3	DP-47	RFNRLSPRRA	V _κ 2	DPK-15	MQALQTR	M	3	1
FTTC-BSA	B05	VH1	DP-25	SSVMGRVPM	V _κ 2	DPK-15	MQALQTLT	Im	3	1
FTTC-BSA	E03	VH3	DP-47	TSGLKSLPRT	V _λ 1	DPL-3	AAWDSSLRLRV	M	3	1
FTTC-BSA	D11	VH3	DP-73	GRGRPSMAYDV	V _λ 1	DPL-3	AAWDSSLALV	Im	4	1
FTTC-BSA	B08	VH3	DP-47	RSGVSLRKYVYI	V _κ 2	DPK-15	MQALQTR	Im	3	1
Plasmin	MP01	VH3	DP-47	MTPQFDY	V _κ 2	DPK-15	MQALQPTT	Im	4	1
Plasmin	MP02	VH4	DP-67	SAYSYFDY	V _λ 3	DPL-16	NSRDSSGQFLV	Im	4	1

Table III. *Continued*

Antigen ^a	Clone	Heavy chains ^b			Light chains ^c			Selection method ^d	Round ^e	No. of copies ^f
		Family	Segment	CDR3 ^g	Family	Segment	CDR3 ^g			
l-PA	MT09	VH1	DP-7	DSGLGDPAL	Vλ2	DPL-11	SSYTSSSTLV	Im	4	1
l-PA	MT03	VH1	DP-7	DSGLGEPAL	Vλ2	DPL-11	SSYTSSSTLG	Im	4	1
l-PA	MT06	VH1	DP-7	DSGLGEPAL	Vλ2	DPL-11	SSYTSSSTLV	Im	4	1
l-PA	MT05	VH1	DP-7	DSGLGEPAL	Vλ2	DPL-11	SSYTSSSTLV	Im	4	1
l-PA	MT07	VH13	DP-7	TSRLKAHPS	Vλ1	DPL-18	QQANSPPITV	Im	4	1
u-PA	MU02	VH13	DP-47	TSRLKAHPR	Vλ2	DPK-15	MRALQITP	Im	4	1
u-PA	MU01	VH13	DP-47	TSRLKAHPS	Vλ1	DPK-8	QQANSPPIT	Im	4	1
u-PA	MU03	VH13	DP-47	TSRLKAHPS	Vλ3	DPK-16	NSRDSSCQLV	Im	4	1
HGF/SF	MH10	VH1	DP-47	GRQSLR	Vλ1	DPK-5	QQANSPPIT	Im	4	1
HGF/SF	MH19	VH1	DP-42	KPFHFGD	Vλ1	DPK-8	QQANSPPIT	Im	4	1
HGF/SF	MH22	VH13	DP-42	KPFHFGD	Vλ1	DPK-5	QQANSPPIT	Im	4	4

^aThe region of the monoclonal antibody NQ11/7.22 (NQ11) bound by the Fab is indicated (Fv or Fc region); three Fabs bound neither fragment and therefore probably interacted with the CH1, Cx or hinge region. Hapten-BSA binding clones listed did not bind BSA alone.

^bHuman germline V_H gene segments are assigned to families as in Tomlinson *et al.* (1992).

^cHuman germline V_λ gene segments (Cox *et al.*, 1994) are assigned to subgroups as in Kabat *et al.* (1991) and human germline V_λ gene segments (Williams and Winter, 1993) are assigned to families as in Chothia *et al.* (1990).

^dIm, selected using antigen-coated immunobeads; M, selected using biotinylated antigen and streptavidin-coated paramagnetic beads. Numbers refer to how many rounds of selection the library had undergone when clones with the sequences indicated were isolated.

^eThe number of independent clones which were isolated with the same sequence.

^fCDR3 (complementarity determining region 3) for both heavy and light chains are as defined by Kabat *et al.* (1991).

^gThese genes appear to have been created by cross-overs between two V genes during PCR amplification.

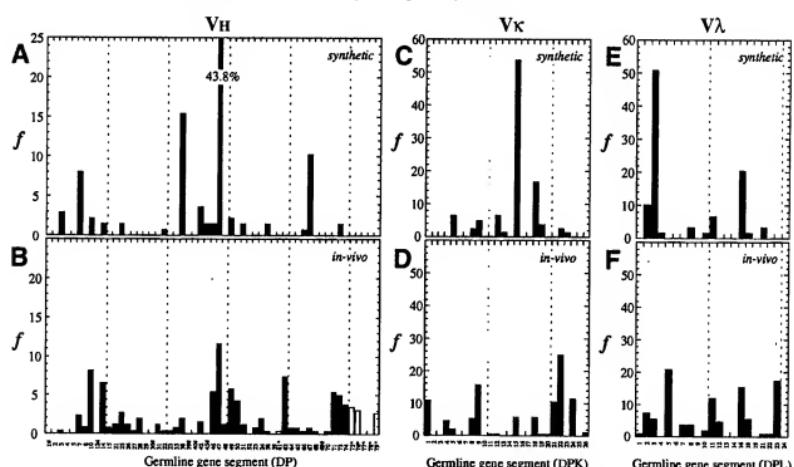


Fig. 4. Use of human germline V gene segments. Frequencies of use of human V_H, V_λ and V_λ segments from the synthetic repertoire (A, C and E), or from natural antibodies (B, D and F). Frequencies (f) are plotted as % of total. V gene usage was compiled for the synthetic antibodies from Table III, and for natural antibodies, from the 292 rearranged V_H genes in the database described in Tomlinson *et al.* (1992), from the 236 rearranged V_λ genes in the database described in Cox *et al.* (1994) and from a database of 110 rearranged V_λ genes taken from the 'Entrez sequence database' (release 8.0; National Center for Biotechnological Information). V_H segments are listed by DP numbers (Tomlinson *et al.*, 1992), V_λ segments by DPK numbers (Cox *et al.*, 1994) and V_λ segments by DPL numbers (Williams and Winter, 1993). All V gene segments listed were included in the synthetic repertoire except those marked (*). V_H gene segments (located on chromosome 15 or 16) which are not used *in vivo* (Tomlinson *et al.*, 1994), but which were included in the synthetic repertoire are indicated (#).

over the limited number of antigens, appears to differ from the usage *in vivo*.

Distribution of CDR3 lengths

We also analysed the lengths and sequences of the CDR3 loops in the selected Fab fragments (Table III; Figure 5).

All the heavy chain CDR3 lengths included in the synthetic repertoire were present. The four residue loops almost all include a glycine residue, presumably to make the tight turn. However, the distribution of the heavy chain CDR3 lengths, biased towards short lengths, contrasts with natural antibodies. This appears to be especially characteristic of

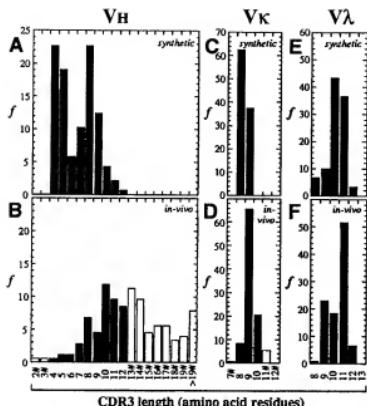


Fig. 5. Distribution of CDR3 lengths. Length distribution of CDR3 loops in human V_H , V_K and V_L chains from the synthetic repertoire (A, C and E), or from natural antibodies (B, D and F). Frequencies (f) are plotted as % of total. Data were compiled as in Figure 4 legend, except that for natural rearranged V_H genes the data was taken from the 177 human genes described by Wu *et al.* (1993). All CDR lengths listed were included in the synthetic repertoire except for those marked (#).

the synthetic Fab fragments binding to haptens-BSA conjugates; those fragments binding to protein antigen appear to have longer CDR3 lengths. The distribution of CDR3 lengths for the synthetic λ light chains was similar to natural antibodies, but differed for the κ light chains, with no loops of 10 residues seen in the synthetic chains.

Pairings of heavy and light chains

The distribution of heavy and light chain pairings (Figure 6) identified some 52 different segment pairings among the 137 unique clones. Several V_H gene segments were found paired with several V_K and V_L gene segments, for example DP-7, DP-38, DP-47 and DP-67 were each found in combination with several different light chain segments. Likewise, the light chain segments DPK-15 and DPL-3 were found in combination with several heavy chain segments. Not surprisingly these segments correspond to those used with higher frequency (see above). The usage of V gene segments differs for antibodies of different specificity, and the pattern of pairings provides a 'fingerprint', readily distinguishing the 'response' to different antigens. Some pairings, for example DP-47 with DPK-15, and DP-47 with DPL-16, were also present in Fab fragments of different specificities.

There were also several examples of 'promiscuous' pairings (Clackson *et al.*, 1991), chains that bind to the same antigen with any of several partner chains (Table III). For example, in the Fab fragments binding to NIP-BSA, the heavy chain sequence (DP-38 segment with CDR3 of sequence AGTL) was paired with six different light chains of V_K 2, V_L 1 and V_L 3 families

(segments DPK-12, DPL-3 and DPL-16, respectively). Likewise in Fab fragments binding to FITC-BSA, the same light chain sequence (DPK-15 segment with CDR3 of sequence MQALQTRT) was paired with 15 different heavy chains of V_H 1, V_H 3 and V_H 4 families (DP-7, DP-47 and DP-67 segments, respectively).

Affinities of selected antibodies

Soluble Fab fragments were produced and purified via their hexahistidine tag by immobilized metal chelate affinity chromatography (IMAC), with typical yields of 100–500 μ g/ml. From the large (6.5×10^{10} clones) repertoire we measured the affinities of several Fab fragments binding to soluble NIP-CAP or fluorescein by fluorescence quench titration (Eisen, 1964). The affinities (K_d) of the Fab fragments ranged from 3.8 to 217 nM (Table IVa). This shows that high affinity anti-hapten antibodies can be isolated directly from large antibody repertoires. We also characterized three Fab fragments binding to the haptens NIP-CAP and fluorescein after selection of a small fraction (10^7 clones) of the repertoire on NIP-BSA or FITC-BSA. In contrast these affinities (K_d) ranged from 0.8 to 12 μ M (Table IVb).

We measured kinetics and affinity of Fab fragments (from the 6.5×10^{10} clone repertoire) binding to immobilized monoclonal antibody NQ11/7.22 and HGF/SF by surface plasmon resonance (Table IVa). For the Fab fragments against the variable region (NML1) and the Fc portion (NML9) of antibody NQ11/7.22, the binding affinities were determined both by on- and off-rate analysis and by Scatchard plots of equilibrium binding (see Figure 7) to be in the range 30–60 nM. For NML1, the on-rate was calculated as $6.4 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and the off-rate as $2.2 \times 10^{-2} \text{ s}^{-1}$; for NML9 as $5.2 \times 10^5 \text{ M}^{-1} \text{ s}^{-1}$ and $3 \times 10^{-2} \text{ s}^{-1}$, respectively. However for the Fab fragment (MH22) against HGF/SF, Scatchard analysis indicated several classes of binding sites, with affinities from micro- to nanomolar. At low Fab concentrations (<40 nM), where high affinity interactions predominate, the affinity could be estimated as 7 nM from an initial on-rate of $1.7\text{--}1.9 \times 10^6 \text{ M}^{-1} \text{ s}^{-1}$ and off-rate of $1.3 \times 10^{-2} \text{ s}^{-1}$.

Discussion

In the immune system, antibodies with moderate affinities are selected from primary repertoires, and their affinities improved step-wise by rounds of somatic mutation and selection. However, theoretical arguments based on the idea of 'shape space' have suggested that larger and more diverse repertoires should give rise to higher affinity antibodies (Perelson and Oster, 1979). The probability (P) that an epitope is recognized by at least one antibody in a repertoire depends on the probability (p) that an antibody recognizes a random epitope with an affinity above a threshold value, and on the number of antibodies (N) according to the equation $P = 1 - e^{-Np}$ (Perelson and Oster, 1979). This predicts, as expected, that the larger the repertoire, the greater the chances of finding a high affinity antibody. However it does not provide an explicit relationship between repertoire size and affinity.

Here we used a repertoire of phage antibodies as an 'artificial immune system' to explore the possibility of

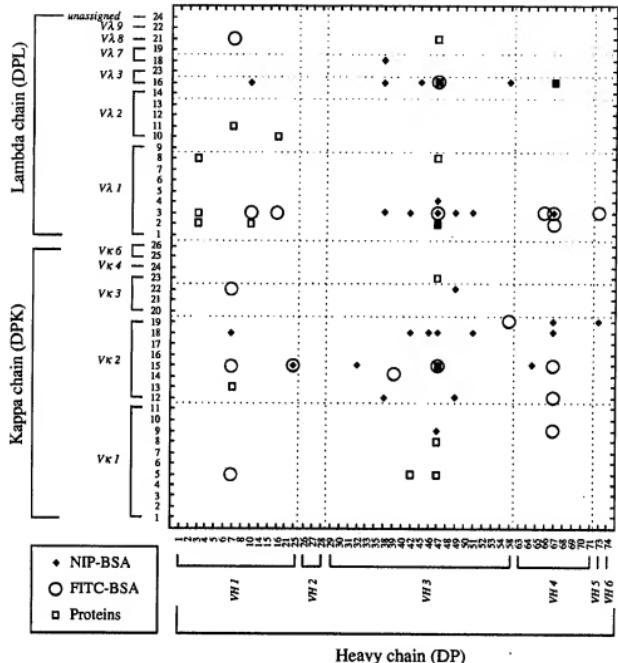


Fig. 6. Pairings of heavy and light chain V gene segments in the synthetic repertoire. Data were compiled and are listed as described in Figure 4 legend. Fab fragments binding NIP-BSA are indicated by black diamonds; Fab fragments binding FITC-BSA by open circles; and Fab fragments binding antibody NQ11/7.22, plasmin, u-PA, I-PA or HGF/SF by open squares.

isolating high affinity antibodies directly from a very large primary antibody repertoire. Previously the size of phage antibody repertoires has been limited to $<10^9$ clones by the efficiency of transfection of DNA into bacteria; here we have used the process of combinatorial infection and *in vivo* recombination (Waterhouse *et al.*, 1993) to overcome this. Bacteria harbouring a repertoire of heavy chains (encoded on a plasmid replicon) were infected with phage encoding a repertoire of light chains, and the heavy chain genes translocated to the phage replicon by recombination within the bacterium. By this means we were able to make a repertoire of 6.5×10^{10} clones and obtained antibodies to a range of antigens and haptens (Table II) with affinities <10 nM (Table IVa). With a smaller repertoire (10^7 clones) we only found antibodies of moderate affinities (>800 nM), in agreement with earlier work in which antibody fragments isolated from smaller repertoires (10^7 – 10^8 clones) were found to have affinities of 700 nM for the hapten NIP (Hoogenboom and Winter, 1992) and 140 nM for the hapten fluorescein

(Barbas *et al.*, 1992c, 1993). The characterization of repertoires of different sizes (and of known diversity) should help in defining the explicit relation between repertoire size and affinity.

As shown in Figure 8, the binding affinities of secondary response (hypermutated) mouse monoclonal antibodies to the haptens NIP and fluorescein were similar to those of human Fab fragments isolated directly from the large repertoire. Only a single mouse anti-NIP antibody has been described (Lucisano-Valim and Lachmann, 1991) with an affinity ($K_d = 5.6$ nM) similar to the best human Fab fragment ($K_d = 4.0$ nM; Table IVa). However, eight mouse monoclonal antibodies have been described with higher affinities for fluorescein than the best human Fab fragment ($K_d = 3.8$ nM; Table IVa), the best of these (4–20) with an affinity of 0.19 nM (Bedzyk *et al.*, 1986).

It is quite possible that antibody fragments with even higher affinities are present in the repertoire, as we had designed the selection process to capture antibody fragments with a range of binding affinities to antigen,

Table IV. Sequences and affinities of Fab fragments

(A) Fabs from 6.5×10^{10} repertoire

Antigen	Clone	Heavy chain		Light chain		K_d (nM)
		segment	CDR3	segment	CDR3	
NIP-CAP	NIP-G6	DP-38	AGTL	DPK-12	MQS IQQLP T	4.0 (± 0.1)
NIP-CAP	NIP-G10 ^a	DP-38	AGTL	DPK-12	MQS IQQLP T	5.4 (± 0.2)
NIP-CAP	NIP-H1 ^a	DP-38	KGSE	DPL-3	AAWDDSLAWF V	11.3 (± 0.4)
NIP-CAP	NIP-C11	DP-38	PGYRGTR	DPL-3	AAWDDSLAS Y	16.5 (± 0.5)
NIP-CAP	NIP-H3 ^a	DP-38	HGH H	DPL-3	AAWDDSLCPEF V	19.7 (± 1.7)
NIP-CAP	NIP-G11 ^a	DP-47	PLNSKKNTTTQ	DPL-3	AAWDDSLF V	20.1 (± 3.6)
NIP-CAP	NIP-G9 ^a	DP-38	AGTL	DPL-3	AAWDDSLV	22.0 (± 1.0)
NIP-CAP	NIP-E5 ^a	DP-38	PNGDQ	DPL-3	AAWDDSLAF V	22.1 (± 0.8)
NIP-CAP	NIP-E7 ^a	DP-38	GGTQ	DPL-3	AAWDDSLV	29.8 (± 1.1)
NIP-CAP	NIP-A4	DP-38	PATRS	DPK-15	M QALQTP L T	48 (± 1.0)
NIP-CAP	NIP-C9	DP-38	GGKD	DPL-18	LLYYGGAY V	59 (± 3.0)
Fluorescein	FITC-B4	DP-66	WSRETNYS	DPL-3	AAWDDSLWS A V	3.8 (± 0.4)
Fluorescein	FITC-A4	DP-67	RSFVG Y EI	DPL-3	AAWDDSLV	14.3 (± 2.0)
Fluorescein	FITC-B11	DP-47	IGQF	DPL-3	AAWDDSLAF V	24.1 (± 0.8)
Fluorescein	FITC-B7	DP-67	AQRKYFDY	DPK-12	M QS I QLRT	151 (± 3.0)
Fluorescein	FITC-A2	DP-47	YRFSSAPPD R	DPL-3	AAWDDSLPS G V	217 (± 16.0)
NQ11 (Fv)	NML1	DP-47	ASSPFV L Q	DPL-21	VLYMGSGSAV	32 ^a and 34 ^b
NQ11 (Fc)	NML9	DP-14	GTGLDG	DPL-10	CSYAGGSYV	41 ^a and 58 ^b
HGF/SF	MH22	DP-42	KFPFH G D	DPK-5	QQANSF P IT	7 ^b

(B) Fabs from 1×10^7 repertoire

Antigen	Clone	Heavy chain		Light chain		K_d (μ M)
		segment	CDR3	segment	CDR3	
NIP-CAP	sNIP-D10	DP-53	PWARGTD	DPK-21	Q QYNNW L ST	8 (± 0.6)
NIP-CAP	sNIP-F3 ^a	DP-47	YNNAADF Y	DPK-21	V LYMGSG H R V	12 (± 1.3)
Fluorescein	sFITC-C2	DP-67	SGV R GLMT	DPK-9	Q QSYSTR T	0.82 (± 0.14)

Affinities (K_d) for haptens were determined by fluorescence quench titration. Affinities (K_d) for protein antigens were determined by SPR. Scatchard analysis^a and from analysis of the rate constants^b. All clones were derived from selections on immunotubes except for those marked^a which were derived from selections using magnetic beads. The residues in the light chain CDR3 regions encoded by randomized codons are in bold.

including those with only moderate affinity. Thus, we took advantage of the binding avidity of multiple Fab fragments on the surface of the phage (by using phage rather than phagemid vectors) and short wash times to retain phage with fast dissociation kinetics. Also for selections on immunotubes we used a high coating density of antigen to favour rebinding of the phage, and for selections with soluble biotinylated antigen we used a relatively high concentration of antigen (50 nM) to try to include even those phage with moderate equilibrium constants (Hawkins *et al.*, 1992). Presumably it would be possible to favour the selection of higher affinity antibodies from this repertoire by more stringent selection.

The phage repertoire was not only large, but also highly diverse. It was assembled from the majority of V gene segments used *in vivo*, including all the major V_H and V_λ families, and V_κ subgroups. The segments included all the major heavy and light chain CDR1 and CDR2 loop conformations (Chothia and Lesk, 1987; Chothia *et al.*, 1989, 1992), and the CDR3 loops were made of diverse sequences and lengths. The chains were paired at random (Huse *et al.*, 1989), creating diverse pairings. The repertoire was sufficiently large that several pairings of a single heavy or light chain could be isolated from the repertoire. Such chain 'promiscuity' is characteristic of repertoires derived from the mRNA from immune sources (see for

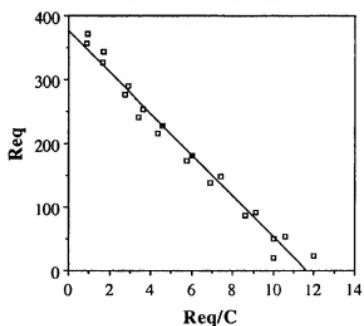


Fig. 7. Affinity of Fab NML1 by SPR. Binding of the Fab to a sensor chip surface coated with mouse monoclonal antibody NQ11/7.22. See Materials and methods for further details. For each concentration of antibody (C, nM), the equilibrium binding signal (Req, in resonance units) was plotted against the Req/C . The slope gives the binding affinity (K_d) as 32.3 nM.

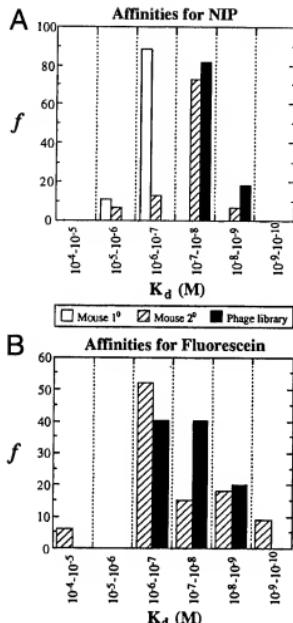


Fig. 8. Affinities for synthetic antibodies and mouse monoclonal antibodies. Affinity data for the Fab fragments from the synthetic repertoire were compiled from Table IVA. (A) Antibodies binding to NIP; data on the mouse immune response were taken from Mariuzza and Strand (1981), Cumano and Rajewsky (1986) and Lucisano-Valim and Lachmann (1991). (B) Antibodies binding fluorescein; data on the mouse immune response were taken from Kranz and Voss (1981), Kranz *et al.* (1982), Reinitz and Voss (1984), Bates *et al.* (1985), Bedzyk *et al.* (1986) and Denzin and Voss (1992).

example Clackson *et al.*, 1991) and reflects the chances of a chain making multiple pairings, which in turn depends on the frequency of the chain and the size of the repertoire.

In the antibodies binding the haptens, the combinations of heavy and light chain segments appeared to be restricted. For example, the antibodies binding to soluble hapten NIP-CAP (Table IVA) mainly utilized the heavy chain segment DP-38 and the light chain segment DPL-3, and included a four residue heavy chain CDR3 loop with a distinctive motif, X-Gly-X-X. This is reminiscent of the restricted response seen with mouse antibodies raised by immunization with the hapten 4-hydroxy-3-nitrophenyl acetate (NP); the immune response in C57BL/6 mice is dominated by antibodies with $\lambda 1$ light chains paired with heavy chains encoded by the V186.2 V_H segment and the DFI16.1 D segment (Bothwell *et al.*, 1981; Cumano and Rajewsky, 1985).

As *in vivo*, only a few segments contributed to most of the 'response'. However, the usage of V gene segments found in the selected Fab fragments from the artificial repertoire differed from that of antibodies from natural repertoires (Figure 4). This might reflect a different representation of segments in the primary repertoire, or the high frequency of sequences from phage antibodies directed against hapten conjugates. Nevertheless we would expect the artificial repertoire to be shaped by different selection pressures to those that operate *in vivo*, leading to a different spectrum of binding sites. If 'holes' exist in the natural repertoire, they may be absent in an artificial repertoire and vice versa. Despite the differences in segment usage between the natural and synthetic antibodies, there is one striking similarity: the heavy chain segment DP-47 is the most commonly used segment in both. *In vivo*, it has been suggested that pre-B cells displaying DP-47 heavy chains are selected by binding to autoantigen (Schwartz and Stollar, 1994). We suggest that the usage of DP-47 reflects a more fundamental property of this segment; the structure of the binding site may be more capable of accommodating a wider diversity of antigens than other segments (Figure 6).

We conclude that human antibodies with affinities in the nanomolar range, and specific for protein antigens and haptens, can be derived directly from large and diverse synthetic phage antibody repertoires. The binding affinities are typical of somatically mutated mouse antibodies produced *in vivo*, and presumably could be improved further through rounds of 'chain shuffling' (Marks *et al.*, 1992b) or point mutagenesis (Hawkins *et al.*, 1992) to create binding specificities and affinities outside the reach of the immune system. In addition, the use of synthetic phage antibody repertoires based on V gene segments to simulate natural immune systems may provide further insights into immune strategy, for example in helping to define the relationship between affinity and repertoire size, or the relationship between the structures of the antigen binding sites and the usage of V , D and J segments, CDR3 lengths and junctional diversity.

Materials and methods

Vectors

The 'acceptor' vector, into which light chain repertoires are cloned, fdDOG-2loxVdel, is identical to fdDOG-2lox (Waterhouse *et al.*, 1993), except that the light chain variable region gene has been deleted (Figure 2A). The 'donor' vector, into which heavy chain repertoires are cloned, pUC19-2loxVdel, is identical to pUC19-2lox (Waterhouse *et al.*, 1993), except that the heavy chain variable region gene has been deleted (Figure 2B). The vector for expression of soluble Fab fragments, pUC19His6mycXba, is a derivative of pUC19 (Veira and Messing, 1987) in which the polylinker has been replaced by the sequence shown in Figure 2C.

Construction of synthetic heavy chain repertoires

A diverse repertoire of rearranged V_H genes has previously been built *in vitro* (Nissim *et al.*, 1994) from a bank of 49 cloned V_H gene segments (Tomlinson *et al.*, 1992) [one of the 50 segments (DP-20) included in the repertoire of Nissim *et al.* (1994) was a pseudogene]. To these segments completely randomized CDR3 regions (Kabat *et al.*, 1991) were appended, varying in size between four and 12 residues (Figure 1A). This cloned repertoire, with $>10^6$ different clones, was re-amplified using PCR with primers pUC-reverse and JH-Xho-FOR (Table VA), the DNA was cut with *Ncol* and *Xhol*, and ligated into pUC19-2loxVdel. The ligation mixture was electroporated (Dower *et al.*, 1988) into *E.coli*

Table V. Oligonucleotides

A. Restoring of synthetic human VH receptors into a UC19-21x VH4c

pJH-Xho-FOR 5'- AGG GGA TAA CAA TTT CAC AGA CG

pJH-Xho-REV 5'- GCG TAA ACC GGC TCC ACC GCG CAA GAC GOT GAC CAG GGT ACC TTT GGC CCA

B. Construction of synthetic human kappa chain repeat(s)

1. Amplification of human Cx

CKPOR 5'- CTG CTA TTA TCG GGC GCG CCT TTA TTA ACA CTC TCC CCT GTT GAA GCT CTT TGT GAC GGG
 CKLink 5'- AGG TTC GGC CAA GCG ACC AAG ATG GAA ATC AAA CTC ACT GTG GCT GCA CCA TGT GTC

2. Human Vx back primers

SYNKB1	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK1,4,5,6,7,9,10
SYNKB2	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK2
SYNKB3	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK3
SYNKB4	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK4,11,12,13,14,15,16
SYNKB5	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK5
SYNKB6	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK6,22
SYNKB7	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK7
SYNKB8	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK8
SYNKB9	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK9
SYNKB10	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK10
SYNKB11	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK11
SYNKB12	5'-CAT GAC GAC CAC ATG GCA CTTG GAC ATG PGG GAG WPG ATG CAA	DPRK12

3. Human Myelinated axons: conduction velocity (CPNS)

DPK11 for primers encoding synthesis CDKs											
DPK11FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GAA	ATT
DPK11REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GTA	ATC
DPK13FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK13REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK14FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK14REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK15FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK15REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK17FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK17REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK18FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK18REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK20FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK20REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK21FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK21REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK22FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK22REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK23FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK23REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK24FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK24REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK26FOR	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC
DPK26REV	5'-	CCT	GGT	CCC	GCC	GAA	CCT	(NNNN)-3'	AAA	GCT	ATC

C. Construction of synthetic human lambda chain repertoires

A model of human aging

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particulars of human CA2
CL2FOR1  5'- GGA ATG CGG GGT GAG GGT TGA GAA
CL2BACK1  5'- CCC CGC AAC GTC TCC CGG TCA TCC
CL2BACK2  5'- GAA TTC CGC GAA GGG ACC AGG CTG ACC GTC CTA GGT CAG CGG AAC AGG GCT GGC CCC TGA GTC GCT ACT
HuCAPORCYSAASNCT  5'- GAA GTC TCA TCC ACT TGA CGC CGC CTG CTA TTA TCC TGA GGC GGC CGT CGT TTA TTA TGA AGA TTC TGT AGG
          GGC CAT TGT CGT

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2. Human V_k back primers

DPV1/1a	5'- CAT GAC CAC ATG GCA CTT CAG TCT GTC TTC AGC CAG CGC CC
DPV1/1b	5'- CAT GAC CAC ATG GCA CTT CAG TCT GTC TTC AGC CAG CGC CC
DPV1/2	5'- CAT GAC CAC ATG GCA CTT CAG TCT GTC TTC AGC CAG CGC CC
DPV1/2a	5'- CAT GAC CAC ATG GCA CTT CAG TCT GTC TTC AGC CAG CGC CC
DPV1/3d	5'- CAT GAC CAC ATG GCA CTT CAG TCT GTC TTC AGC CAG CGC CC
DPV1/7	5'- CAT GAC CAC ATG GCA CTT CAG TCT GTC TTC AGC CAG CGC CC
DPV1/9	5'- CAT GAC CAC ATG GCA CTT CAG TCT GTC TTC AGC CAG CGC CC

3. Human V λ forward primers encoding synthetic CDR3s

D. Sub-cloning of selected repertoires for expression of soluble Fab fragments

5'- GAA TTT TGT GTA TGA GG
 5'- GTC TCC GCA ACT TCC TTA AGA CAA TTT CAC AGT AAG GAG GTT TAA CTT GTG AAA AAA TTA TTA TTC GCA ATT

E. Sequencing and probing

INFR3DR3P	5'-GCT TGG AAC GCA GCA GCG
CTA lib.seq	5'-GCA GTC TTC GCT TGG GCT TGA AGC
CH1 lib.seq	5'-GGT GCT CTT GCG GCG GCG TGC
petRock	5'-GAA ATA CCT ATT GCG GCG TGC GG
petRock	5'-GCG AGG GCG GCT GTC ATT GTC GCG
dpCR3Rock	

A = adenosine; C = cytosine; G = guanine; T = thymidine; Y = C or T; R = A or G; W = A or T; S = G or C; K = T or G; M = C or A. Restriction sites are underlined. Primer names are to the left of the sequences and the V-gene segments amplified to the right.

TG1 (Gibson, 1984) to create the library pUC19-2loxVHlib, and in total 5×10^6 clones were obtained. Diversity was confirmed by *Bst*NI fingerprinting (Clackson *et al.*, 1991) and sequencing of 24 independent clones. All these clones were found to be different.

Construction of synthetic kappa chain repertoires

The human C_x gene was amplified from the vector pSW1/FabD1.3 (Skerra *et al.*, 1991) by PCR with Taq polymerase using primers CxFOR and CxLink (Table VBl) which introduce a consensus human J_x segment at the 5'-end of the C_x gene and two stop codons (TAA) and an Acl site at the 3'-end. The reaction mixture (50 μ l) was cycled 25 times (94°C for 1 min, 60°C for 1 min, 72°C for 1 min). The amplified C_x gene was purified using Magic PCR Preps (Promega) and resuspended in 50 μ l water.

In parallel, 26 human germline V_x gene segments with open reading frames (DPK1-26), which had been cloned from the genomic DNA of a single individual (Cox *et al.*, 1994), were individually amplified with Back primers that introduce an ApaI site at the 5'-end (see Table VB2) and Forward primers that append a portion of the J_x segment to the 3'-end (see Table VB3). For each segment three independent PCRs were performed with different Forward primers to construct CDR3 regions (Kabat *et al.*, 1991) of length eight, nine or 10 residues that included one, two or three residues of random sequence (Figure 1B). For each gene, a 50 μ l PCR was performed using Taq polymerase and toothpicked frozen glycerol stocks of *E. coli* infected with the appropriate M13 clone as template. Reactions were cycled 25 times (94°C for 1 min, 60°C for 1 min, 72°C for 1 min). The Forward primers (Table VB3) introduced length and sequence diversity into CDR3 corresponding to that observed *in vivo* (Kabat *et al.*, 1991).

The amplified V_x genes were each joined to the amplified C_x gene using PCR (Horton *et al.*, 1989). Assembly PCRs (25 μ l) used Taq polymerase, 1 μ l of amplified C_x and 0.8 μ l of the V_x gene PCR from above. The appropriate V_x Back primer was used for each gene (Table VB2) together with C_x FOR (Table VB1) and the reaction cycled 30 times (94°C for 1 min, 55°C for 1 min, 72°C for 2 min).

The PCR assembly reactions for each V_x gene were checked by agarose gel electrophoresis, then pooled with the other V_x genes according to CDR3 length, and the V_x-C_x genes purified using Magic PCR Preps. The pooled DNA was then cut with ApaI and AclI and digested DNA (~6 μ g) purified from a 1.5% low melting-point agarose gel using Magic PCR Preps. Approximately 1 μ g of the purified and cut V_x-C_x DNA from each pool was ligated in a 60 μ l volume with 1200 U of T4 DNA ligase (New England Biolabs) to ~5 μ g of digested fdDOG-2loxVHdel vector [previously electro轉 from a 0.8% agarose gel (Sambrook *et al.*, 1990)]. DNA was purified from the ligation mixture using GeneClean II (BIO 101), resuspended in 30 μ l water, and electro轉 (Dower *et al.*, 1988) into four 50 μ l aliquots of *E. coli* TG1. Cells were grown in 1 ml 2XY broth containing 1% glucose for 1 h and then plated in 243 mm² 243 mm dishes (Nunc) on TYE (Miller, 1972) medium with 2.5 μ g/ml tetracycline (TYE-TET). After overnight incubation at 37°C, colonies were scraped off the plates into 7 ml of 2XY broth (Miller, 1972) containing 15% (v/v) glycerol for storage at -70°C.

The frequency of inserts was checked by PCR for each of the three pools and found to be 90% for CDR3 of eight residues, 100% for CDR3 of nine residues and 87% for CDR3 of 10 residues. The number of clones with light chains could then be calculated as 9.9 \times 10³ (CDR3 of eight residues), 1.5 \times 10⁴ (CDR3 of nine residues), 6.5 \times 10⁴ (CDR3 of 10 residues). Sequence diversity was confirmed by sequencing eight clones of each CDR3 length; all clones were found to be different. The pools were then combined, to create the library fdDOG-2loxVHlib, corresponding to 9.0 \times 10⁶ light chains.

Construction of synthetic lambda chain repertoires

The human C_{A2} gene (Vasicek and Leder, 1990) was amplified from genomic DNA by PCR with Taq polymerase using primers CL2BACK1 and CL2FOR1 (Table VBl) based in the regions flanking the C_{A2} exon. The EcoRI and HindIII sites in CL2FOR1 and CL2BACK1, respectively, were used to clone the PCR product into M13mp19 (Yanisch-Perron *et al.*, 1985).

Twenty-one V_A germline gene segments with open reading frames, previously cloned in M13mp19 (Williams and Winter, 1993), were individually amplified using PCR and Taq polymerase with back primers (Table VC2) which anneal to framework 1 (FR1) and introduce a 5' ApaI site, and forward primers (Table VC3) which append a portion of the J_{A2} gene (Vasicek and Leder, 1990) to CDR3. CDR3 loops (Kabat *et al.*, 1991) of eight to 13 residues that included zero, one, two, three,

four or five residues of random sequence were encoded by the forward primers (Figure 1C). The number of residues of random sequence included was designed to match the pattern of V gene rearrangement seen in *vivo* and varied with the different V_A segments used.

Each synthetically rearranged V_A gene was individually joined to the human C_{A2} gene by PCR with Taq polymerase (Horton *et al.*, 1989). Each 50 μ l PCR assembly reaction contained -1 ng of M13mp19 containing the C_{A2} gene, -0.1 μ g of the V_A gene, the appropriate (FR1) back primer (25 pmol) (Table VC2), the back primer CL2BACK2 (2.5 pmol) (Table VC1), which contains the 3' sequence of the J_{A2} gene linked to the 5' sequence of the C_{A2} gene, and the forward primer HUCAFORCYASCNOT (25 pmol) (Table VC1) which appends two stop codons (TAA) followed by an Acl site to the 3' of the C_{A2} gene. Reactions were cycled 30 times (94°C for 1 min, 65°C for 1 min, 72°C for 2 min).

The PCR assembly reactions were combined into a single pool and the human V_A genes digested and ligated into fdDOG-2loxVHdel as described for the V_x-C_x gene pools (see above), thus creating the library fdDOG-2loxVHlib. Ninety-two percent of clones were found to carry inserts of the correct size, corresponding to a repertoire size of 7.4 \times 10⁶ light chains. Thirty-three clones were sequenced to confirm the presence of each V_A segment. All the sequences were different.

Combinatorial infection and *in vivo* recombination

To create a large combinatorial repertoire of heavy and light chains on an fd phage vector we used the strategy of combinatorial infection and *in vivo* recombination (Waterhouse *et al.*, 1993). This system uses the *lox*-Cre site-specific recombination system of bacteriophage P1 (Stemberg and Hamilton, 1981; Hoess *et al.*, 1982) to bring together heavy and light chain genes onto the same replicon.

Phage P1 lysates were made by thermal induction (Rosser, 1972). *E. coli* C600 Su (Appleyard, 1954) harbouring phage P1Cm1.100 ℓ^{-1} m⁻¹ (Yarmolinsky *et al.*, 1989) were grown in a 2 l baffled flask containing 1 l of 2XY, 25 μ g/ml chloramphenicol, 10 mM MgSO₄ with vigorous shaking at 30°C to OD₆₀₀ of 0.6. The temperature was then raised quickly to 42°C by shaking in a 70°C water bath and then shaking continued for a further 35 min in a 40°C water bath. Shaking was then continued at 37°C until lysis was visible (usually 1.5–2 h). The culture was then spun at 5000 g for 15 min at 4°C and 100 μ l chloroform added to the supernatant. P1 phage titres were measured by adding serial dilutions of the lysate to mid-log phase *E. coli* TG1 (Gibson, 1984) grown in 2XY broth containing 5 mM CaCl₂, the mixture incubated for 30 min at 30°C to allow infection and then plated on TYE medium (Miller, 1972) containing 30 μ g/ml chloramphenicol. Chloramphenicol resistant colonies were counted after 24 h incubation at 37°C. The P1 titre of the lysate used for this library was 3 \times 10⁹ transducing units (t.u.) per ml.

10⁶ *E. coli* TG1 harbouring the library of synthetic V_x genes (or the library of synthetic V_A genes) cloned in fdDOG-2loxVHdel, were used to inoculate 1 l of 2XY broth containing 12.5 μ g/ml tetracycline (2X TY-TET) and the culture shaken for 20 h at 30°C in two 500 ml aliquots in 2 l baffled Erlenmeyer flasks. Phage were purified from the supernatant by precipitation with polyethylene glycol as in McCafferty *et al.* (1990), resuspended in PBS (phosphate buffered saline: 25 mM Na₂PO₄, 125 mM NaCl, pH 7.0) and filtered through a 0.45 μ m sterile filter (Minitar, Sartorius). Phage were titred by infecting exponential phase *E. coli* TG1 (30 min, 37°C) and plating on TYE-TET. Yields were typically 10¹⁰ t.u. per ml of culture.

At various points during the recombination procedure aliquots of bacteria were removed and serial dilutions plated on TYE plates supplemented with 1% glucose and containing a variety of different antibiotics (100 μ g/ml ampicillin; 15 μ g/ml tetracycline; 30 μ g/ml chloramphenicol). From the number of colony forming units (c.f.u.) the overall repertoire size could be calculated. These points are indicated in the protocol below and the results are summarized in Table I (also see Results).

Approximately 10⁹ *E. coli* TG1 harbouring the library of synthetic heavy chain genes cloned in pUC19-2loxVHdel (pUC19-2loxVHlib; see above) were used to inoculate 100 ml 2XY broth containing 100 μ g/ml ampicillin and 1% (w/v) glucose (2X TY-AMP-GLU). An aliquot of bacteria was plated for c.f.u. determination (see Table I, sample point 1) and the rest of the culture grown overnight at 30°C. An aliquot of bacteria was then plated for c.f.u. determination (see Table I, sample point 2). Two 5 ml aliquots of the overnight culture were then used to inoculate two 500 ml aliquots of 2X TY-AMP-GLU in 2 l Erlenmeyer flasks and the cultures grown, shaking, at 37°C to an OD₆₀₀ of 0.5.

2 \times 10¹² t.u. of V_A library in fdDOG-2loxVHdel were added to one

of the above cultures and 2×10^{12} t.u. of V_λ library in fdDOG-2loxVkb1 were added to the other culture. Each culture was immediately split into 5×100 ml aliquots and each aliquot mixed with 1 l of 2 \times TY-AMP-GLU, pre-warmed to 37°C. These cultures were then incubated at 37°C, without shaking for 30 min, and then with shaking until an OD_{600} of 0.4 was reached (~30 min). An aliquot of bacteria from the kappa infection and another from the lambda infection were plated for c.f.u. determination (see Table I, sample point 3).

$CuCl_2$ was then added to a final concentration of 5 mM and 200 ml phage P1/Cm \times 1.10 \times 10 1 lysate (6×10^{11} t.u. see above) were added to each 1 l flask (giving a multiplicity of infection of ~1). Incubation was continued at 30°C for 1 h, with a short burst of shaking every 15 min. The culture was then centrifuged at 5000 g for 15 min and the pellets resuspended in the original volume of 2 \times TY broth containing 100 μ g/ml ampicillin, 12.5 μ g/ml tetracycline, 25 μ g/ml chloramphenicol and 1% glucose (i.e. 5 l for the V_λ library and 5 l for the V_κ library). An aliquot of bacteria was plated for c.f.u. determination (see Table I, sample point 4). The repertoire size was determined to be 6.5×10^{10} from the number of ampicillin, tetracycline and chloramphenicol resistant c.f.u. at this point (see Results). An aliquot of each culture was also centrifuged at 12 000 g for 5 min and the supernatant filtered through a 0.45 μ m sterile filter (Minisart, Sartorius). The fd phage in the supernatant were titered by infecting exponential phase *E. coli* TG1 (30 min, 37°C) plating on TYE-TET (see Table I, sample point 5).

The cultures were incubated overnight at 30°C with shaking for 24 h in 2 l baffled flasks (1 l medium per flask). An aliquot of bacteria from each culture was plated for c.f.u. determination (see Table I, sample point 6). The fd phage in the supernatant were also titered by infection of exponential phase *E. coli* TG1 as above (see Table I, sample point 7). The total yield of fd phage was 4.1×10^{11} t.u. and therefore >99.9% were propagated from bacteria containing the pUC 'donor' vector and phage P1. The cultures were then centrifuged at 5000 g for 15 min at 4°C and the fd phage precipitated from the supernatant using polyethylene glycol (McCafferty *et al.*, 1990) and resuspended in a final volume of 10 ml PBS.

Ten 2 l flasks, each containing 1 l 2 \times TY broth were inoculated with *E. coli* TG1 and grown, shaking, at 37°C until an OD_{600} of 0.4 (~4 \times 10 12 bacteria) was reached. Two microlitres of the above recombinant V_λ fd phage (8×10^{11} t.u.) were added to 5 l of *E. coli* and 2 ml of the above recombinant V_λ fd phage (2×10^{12} t.u.) were added to the other 5 l of *E. coli* and the cultures held at 37°C for 30 min without shaking and then for 30 min with shaking. The number of *E. coli* infected with fd phage was determined by plating bacteria on TYE-TET plates to be 1.7×10^{12} (V_λ repertoire) and 1.1×10^{12} (V_κ repertoire). This exceeds the estimated repertoire size by >10-fold, hence maintaining library diversity. Tetracycline was then added to 12.5 μ g/ml and the culture shaken for 16 h at 30°C. The V_λ culture and the V_κ cultures were then centrifuged at 5000 g for 10 min and the pellet from each repertoire resuspended in 250 l 2 \times TY broth containing 15% glycerol and stored in 15 ml aliquots at -70°C.

Aliquots of the two libraries were also spread on TYE-TET in 243 mm \times 243 mm dishes (Nunc). After overnight incubation at 30°C the number of colonies on the large plates were calculated from the number of colonies on small TYE-TET plates on which serial dilutions had been spread. Two plates, one containing 3.5×10^6 colonies of the V_λ library and the other containing 6.4×10^6 colonies of the V_κ library were selected, and the bacteria scraped into 10 ml 2 \times TY broth containing 15% glycerol. This stock therefore corresponded to a repertoire of 10 7 clones.

Assaying the efficiency of *in vivo* recombination

To test the efficiency of replacement of the anti-TNF heavy chain in the 'acceptor' vectors (fdDOG-2loxVkb1 and fdDOG-2loxVkb1b) with synthetic heavy chain from the 'donor' vector (pUC19-2loxVkb1b), 250 individual colonies from each of the V_λ and V_κ recombinant libraries were picked onto TYE-TET plates and grown overnight at 30°C. Colony hybridization was then performed as in Tomlinson *et al.* (1992), with a primer (TNFCDR3PRB; Table VE) complementary to the CDR3 region of the anti-TNF heavy chain gene found in the 'acceptor' vectors (fdDOG-2loxVkb1 and fdDOG-2loxVkb1b). Where recombination is successful, the anti-TNF heavy chain gene should be replaced by the synthetic heavy chain from the 'donor' vector. Probing of the colonies indicated that only 12 V_λ colonies (5%) and 39 V_κ colonies (16%) retained the original heavy chain. Probing of 250 colonies from each of the V_λ and V_κ phage libraries before recombination indicated that, as expected, all colonies harboured the original heavy chain.

Clones lacking the anti-TNF heavy chain gene ($42 V_\lambda$, $48 V_\kappa$) were

screened by PCR (Güssow and Clarkson, 1989) for the presence of heavy chains with the primers pELB/ck and CH1.lib.seq (see Table VE) and for the presence of light chains with the primers fdPCRback and CK.lib.seq (or CL.lib.seq). The probing and PCR screening indicated that in the recombinant V_λ library, 28% of clones had acquired a heavy chain from the donor vector and also had a lambda light chain gene; 5% were unrecombined fdDOG-2loxVkb1b; and 67% had deletions of the light chain, heavy chain or both. For the V_κ library, 28% of clones had acquired a heavy chain from the donor vector and also had a kappa light chain gene; 16% were unrecombined fdDOG-2loxVkb1b; and 56% had deletions of the light chain, heavy chain or both. Further cycles of infection (without selection) led to a further decrease in the frequency of phage harbouring heavy and light chain genes, presumably due to competition with deletion phage. Nevertheless, immediately after recombination, for both V_λ and V_κ repertoires, we can calculate that 28% of all fd phage clones had both heavy and light chain genes and that the heavy chain gene derived from the pUC 'donor' vector.

Propagation of phage from the recombinant library

Five litres of 2 \times TY-TET were inoculated with a 15 ml aliquot of the recombinant V_λ library glycerol stock (5×10^{11} c.f.u.) and a further 5 l 2 \times TY-TET inoculated with a 15 ml aliquot of the recombinant V_λ library glycerol stock (1×10^{11} c.f.u.). The cultures were grown shaking, overnight at 30°C in baffled flasks (1 l medium per flask). The cultures were centrifuged at 5000 g for 15 min at 4°C and the fd phage precipitated from the supernatant using polyethylene glycol as in McCafferty *et al.* (1990) and each repertoire resuspended in a final volume of 10 ml PBS. Total phage yields (from 10 l) were typically around 10^{14} t.u.

Selection of the recombinant library on immunobots

The phage repertoire was panned using immunobots (Nunc; Maxisorp) coated with each antigen (Marks *et al.*, 1991; Griffiths *et al.*, 1993). A range of antigens was used, as described in Table II. Here we have focused on *live* protein antigens: a mouse monoclonal antibody (NQ11/7.22; Griffiths *et al.*, 1984), and four proteins belonging to the kringle-serine proteases family (HGF/SF, plasmin, t-PA and u-PA). In addition, selection was performed on two haptens conjugated to BSA: (FITC Isomer I and NIP). FITC conjugated to BSA (FITC-BSA; 11.2 FITC groups per BSA molecule) was purchased from Sigma; NIP conjugated to BSA (NIP-BSA) was synthesized by coupling NIP-caproate-O-succinimide to BSA (Brownstone *et al.*, 1966) to give 27.9 NIP groups per BSA molecule. Tubes were coated with 10 μ g/ml protein or 100 μ g/ml hapten-BSA conjugates in PBS overnight at room temperature.

For the first round of selection 0.5 ml (6.4×10^{12} t.u.) of the recombinant V_λ library and 0.5 ml (7.5×10^{12} t.u.) of the recombinant V_κ library were used per immunobot. For the first two rounds of selection tubes were washed 10 times with PBS, 0.1% (v/v) Tween 20 and 10 times with PBS. For subsequent rounds of selection tubes were washed 20 times with PBS, 0.1% (v/v) Tween 20 and 20 times with PBS (Griffiths *et al.*, 1993). Phage were eluted with 100 mM triethylamine (Marks *et al.*, 1991). Eluted phage were used to infect 10 ml log phase *E. coli* TG1 cells and plated on TYE-TET medium in 243 \times 243 mm dishes (Nunc). After incubation overnight at 30°C the colonies were scraped off the plate into 200 ml 2 \times TY-TET and incubated, shaking, at 30°C for ~6 h. The culture was centrifuged at 5000 g for 15 min at 4°C and the fd phage precipitated from the supernatant using polyethylene glycol (McCafferty *et al.*, 1990), each repertoire being resuspended in a final volume of 2 ml PBS. The cell pellet was resuspended in 20 ml 2 \times TY broth containing 15% glycerol and a 2 ml aliquot stored at -70°C.

One millilitre of these phage (~10 12 t.u.) was used per immunobot for the next round of selection. The library was subjected to four or five rounds of growth and selection for each antigen.

Selection of the recombinant library using streptavidin-coated paramagnetic beads

The library was also selected using soluble biotinylated hapten-BSA conjugates and streptavidin-coated paramagnetic beads (Hawkins *et al.*, 1992), but with some modifications. FITC-BSA (11.2 FITC:BSA) and NIP-BSA (27.9 NIP:BSA) (see above) were biotinylated using Immunopure NHS-SBiotin (sulfosuccinimidyl 2-(biotinamido)ethyl-1,3-dithiopropionate; Pierce) according to the manufacturer's instructions.

For the first round of selection 0.5 ml (6.4×10^{12} t.u.) of the recombinant V_λ library and 0.5 ml (7.5×10^{12} t.u.) of the recombinant V_κ library were made up to 2.5 ml with PBS and mixed with 2.5 ml PBS containing 4% skimmed milk powder, 50 μ l Tween 20, and biotinylated hapten-BSA added to give a final concentration of 50 nM. The mixture

was then gently rotated on an inclined wheel for 1 h at room temperature. 1.5 ml of Dynabeads M-280 coated with Streptavidin (Dynal) (and previously blocked by incubating for 2 h at 37°C with PBS containing 2% skimmed milk powder (2% MPBS)) were then added and mixing continued for a further 15 min at room temperature. The Dynabeads were then washed a total of 15 times, using a Dynal MPC (Magnetic Particle Concentrator); each wash was with 1 ml PBS or with 1 ml 2% MPBS (every third wash). Phage were eluted from the beads by incubating 5 min at room temperature in 300 µl PBS, 50 mM dithiothreitol (DTT) and the eluate used to infect 10 ml log phase *E.coli* TG1 cells and plated onto TYE-TET in 243×243 mm dishes (Nunc). Phage were harvested from the plates as above and each repertoire resuspended in a final volume of 2 ml PBS. The cell pellet was resuspended in 20 ml 2×TY broth containing 15% glycerol and a 2 ml aliquot stored at -70°C (see above).

For the second round (and subsequent rounds) of selection 1 ml of phage ($\sim 10^{10}$ t.u.) were mixed with 0.5 ml PBS containing 6% skimmed milk powder, 10 µl Tween 20, and biotinylated haptens-BSA added to give a final concentration of 50 nM. Selection was then as above, except that only 300 µl of streptavidin-coated Dynabeads M-280 were used. The library was subjected to four or five rounds of growth and selection for each antigen.

ELISA screening of repertoire selections

'Polyclonal' mixtures of phage produced by re-propagation of the library after each round of selection were screened for binding to the antigen used for selection and to other control antigens by ELISA. The phage ELISA was performed essentially as McCafferty *et al.* (1990) using 10 µl PEG precipitated phage ($\sim 10^{10}$ t.u.), but using horseradish peroxidase conjugated anti-sheep antibody (Sigma) and 3',3',5',5'-tetramethylbenzidine (TMB) for detection. Reactions were stopped by the addition of H₂SO₄ after 10 min and readings taken by subtracting the A₅₅₀ from the A₄₅₀. All antigens were coated at 10 µg/ml in PBS.

Single tetracycline resistant colonies from infection of *E.coli* TG1 with eluted phage were also screened to identify those producing antigen-binding phage by ELISA essentially as in Clackson *et al.* (1991) except that phage were grown at 30°C, and detection was as for the polyclonal phage ELISA above.

Sub-cloning of selected repertoires for expression of soluble Fab fragments

Approximately 10⁸ bacteria harbouring phage fd were taken from the stocks frozen down after the appropriate round of selection (5 µl of a 10-fold dilution of the frozen stock). In general, these bacteria were used as template in a 50 µl PCR reaction and the heavy chain genes amplified by pre-soaking at 94°C for 10 min and then cycling 30 times (94°C for 1 min, 50°C for 1 min, 72°C for 2.5 min) using the primers fdSEQ1 and G3LXbaGTGBack (Table VI). The products were run on a 1.3% low melting point agarose gel and purified from the gel using 'Magic PCR Prep' (Promega). The DNA was then cut with *Xba*I and *Nor*I and ligated into pUC119His6mycXba. The ligation mixture was electroporated (Dower *et al.*, 1988) into *E.coli* TG1 and plated onto TYE medium containing 10 µg/ml ampicillin and 1% glucose (TYE-AMP-GLU) and incubated at 37°C overnight.

Individual ampicillin resistant colonies were grown in 96-well plates and soluble Fab production induced with isopropyl β-D-thiogalactoside (IPTG) as in Marks *et al.* (1991). Soluble Fab fragments in supernatants were assayed for binding to antigen coated plates by ELISA. All antigens were coated at 10 µg/ml in PBS. Bound Fab's were detected with a mixture of rabbit anti-human λ light chain antibodies and rabbit anti-human κ light chain antibodies (Dako) followed by goat anti-rabbit IgG (whole molecule) peroxidase conjugate (Sigma), or by using a mixture of peroxidase conjugated sheep anti-human κ light chain (free and bound) antibodies and peroxidase conjugated sheep anti-human λ light chain (free and bound) antibodies (The Binding Site). ELISAs were developed with TMB as above.

Each antigen-binding clone was streaked on a TYE-AMP-GLU plate and two individual colonies picked and re-assayed for production of antigen-binding Fab fragments as above. Positive clones were stored in 2×TY, 15% glycerol at -70°C.

Sequencing of antibody V genes

Clones, toothpicked from frozen glycerol stocks, were amplified by PCR using Tag polymerase. Reactions (50 µl) were pre-soaked for 10 min at 94°C and then cycled 25 times (94°C for 1 min, 55°C for 1 min, 72°C for 30 s.). The primers were Cx.lib.seq and LMB3 for kappa chain

amplification; Cλ.lib.seq and LMB3 for lambda chain amplification; and CH1.lib.seq and pellBlack for heavy chain amplification (Table VE). Aliquots of the product were analysed on a 1.3% agarose gel. The remaining product was purified using Magic PCR Preps (Promega).

PCR cycle sequencing reactions with fluorescent dideoxy chain terminators (Applied Biosystems) (20 µl in 25 cycles; 96°C for 30 s, 50°C for 15 s, 60°C for 4 min) were carried out according to the manufacturer's instructions. The purified PCR product from above (200 ng) was used as template with the following primers: Cx.lib.seq for kappa chains, Cλ.lib.seq for lambda chains and CH1.lib.seq for heavy chains (Table VE).

The sequencing reactions were analysed on an Applied Biosystems 373A Automated DNA Sequencer. Sequence analysis was performed using SeqEd (Applied Biosystems) and MacVector (IBI Kodak, New Haven, CT).

Epitope mapping of Fab fragments binding monoclonal antibody NQ11/7.22

The repertoire selected on the mouse monoclonal antibody NQ11/7.22 (Y.I.C., Griffiths *et al.*, 1984) was sub-cloned after the second, third and fourth round of selection for production of soluble Fab fragments. 161 out of 384 clones bound to NQ11/7.22 (see above), and were further mapped by binding to MOPC21 (an unrelated mouse γ1 κ monoclonal antibody); Sigma; mouse Fc fragment (Jackson); and NQ11/7.22 zero-linker diabody (containing only the heavy and light chain variable domains; Holliger *et al.*, 1993). All proteins were coated at 10 µg/ml in 50 mM NaHCO₃ (pH 8.3). Eleven different Fab fragments identified by sequencing (Table III) were thereby directly mapped to binding to the Fv (idiotypic) or Fc regions, or to the remaining portions of the monoclonal antibody.

Purification of Fab's and affinity determination

Several pUC119His6mycXba clones encoding NIP and fluorescein conjugated Fab fragments were chosen at random for affinity determination. A 1 l culture of *E.coli* TG1 (Gibson, 1984) harbouring each plasmid was grown and Fab expression induced with IPTG (De Bellis and Schwartz, 1990). After induction, the culture was shaken for 3 h at 25°C and the Fab fragments harvested from the periplasm, essentially as in Breitling *et al.* (1991).

The antibodies were purified by IMAC (Hochholz *et al.*, 1988; Hoffmann and Roeder, 1991). The pooled 'periplasmic fraction' and 'oxmucoid shock fraction' were passed over a 5 ml Ni-NTA resin (Dagen) according to the manufacturer's instructions. The column was washed with 50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl, 33 mM imidazole and the protein was eluted by applying 50 mM sodium phosphate buffer pH 7.5, 500 mM NaCl and 100 mM imidazole. The eluted protein was dialysed against 2× 3 l PBS for 24 h. The dialysed fractions were then analysed by SDS-PAGE (Laemmli, 1970) under non-reducing conditions and the concentration determined spectrophotometrically (assuming A₂₈₀ of 1.0 = 0.7 mg/ml).

Affinities of the purified Fab's were determined by fluorescence quench titration with free hapten (Eisen, 1964), essentially as described by Foote and Milstein (1991). The hapten used were fluorescein (Sigma) or NIP-CAP. All measurements were made with a Hitachi F-4500 spectrophotometer, using an excitation wavelength of 280 nm and monitoring emission at 340 nm. Antibody (1.0 ml) in PBS was placed in a 10 mm×10 mm cuvette in the instrument, mixed continually using a magnetic stir-bar, and held at 20°C. Hapten additions were made automatically using a 50 µl gas-tight syringe (Hamilton) driven by a MicroLab M syringe controller (Hamilton). The titrations and data collection were performed automatically using a Dell 433L computer interfaced with the syringe controller (through an RS-232 interface) and the spectrophotometer (through an IEEE interface and an instrument driver supplied by the manufacturer). The computer was programmed in QuickBasic (Microsoft). Data were averaged from two to five runs.

The binding of three of the Fab fragments binding protein antigens were analysed by SPR using the BIACore system (Pharmacia Biosensor). The active concentrations of purified Fab fragments NML1 (anti-NQ11/7.22 Fv), NML9 (anti-NQ11/7.22 Fc) and MH22 (anti-HGF/SF) were determined by the measurement of mass transport-limited binding slopes (Karlsson *et al.*, 1993). The antibody NQ11/7.22 was purified from ascites by affinity chromatography (Mäkitalo *et al.*, 1978) and 990 resonance units (RU) immobilized on the biopchip by chemical coupling with NHS/EDC (Johnston *et al.*, 1991; Chaiken *et al.*, 1992). HGF/SF was immobilized on the biopchip after biotinylation. Thus 3.8 kRU of immobilized streptavidin (O'Shannessy *et al.*, 1992) were used to capture

2800 RU HGF which had been biotinylated with biotin-LC-hydrazide (Pierce) after periodate oxidation of the carbohydrate using a protocol based on O'Shaughnessy (1990), but with 20 mM Na₂SO₄ to quench excess periodate (Weber and Hof, 1975). After capture the hydrazide bond between the biotin and the HGF was stabilized by reduction with sodium cyanoborohydride (0.1 M in 0.1 M Na acetate pH 4; 40 µl at 2 µl/min). The fragments were passed over the surface at 5 µl/min at 25°C in concentrations ranging from 2 to 400 nM, and the dissociation constant determined by Scatchard analysis of the equilibrium binding, and on- and off-rate constants by a kinetics analysis software (Pharmacia Biosensor) (Karlsson *et al.*, 1991; Chaiken *et al.*, 1992).

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